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The ecology of nitrite-oxidizing bacteria in grassland soils

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In order to enable the study of the spatial and temporal distribution of nitrite-oxidizing bacteria in semi-natural grassland soils, it was necessary to find a reliable method for enumerating these bacteria. The MPN-technique seemed the most suitable to determine the total number of viable nitrite oxidizers.

However, it appeared (Chapter 2) that the results of the MPN-technique were very much dependent on the nitrite concentration applied in the incubation medium. In the water saturated peat soil of Merrevliet highest numbers were always obtained at low nitrite concentrations, whereas high nitrite concentrations mostly yielded the highest counting results in the well drained, slightly acid sandy soil of Ommen. In another soil type (Brummen), the nitrite concentration did not influence the outcome of the MPN-enumerations in most of the samples. It was concluded that the MPN-enumeration technique could only be applied if more than one nitrite concentration was used.

To determine the temporal variation in the size of the nitrite-oxidizing community it was necessary to find out how a field-plot should be sampled in order to get a representative estimation of the size of the community in the whole plot. Based on the results obtained from different sampling locations which are described in Chapter 2, the plot *Ommen-top* was chosen for further study. The dependency of the MPN-results on the nitrite concentration in the incubation medium seemed to be a characteristic of the nitrite-oxidizing community in a certain soil type at the time of sampling. Because understanding of this behaviour in the MPN-incubations could lead to more information about the nitrite oxidizers than only their most probable number, an effort was made to explain the phenomena observed.

In Chapter 3, the hypothesis was tested that dormant nitrite-oxidizing cells were activated in the MPN-incubation only at high nitrite concentrations, while remaining inactive at a low nitrite concentration. However, this could not be confirmed. Actively growing cells sampled from the culture vessel and from the effluent vessel of nitrite limited chemostats, even after prolonged resting periods of 1.5 years, were enumerated using different techniques. All cells proved to be viable at both nitrite concentrations tested. However, the counting results in the two incubation media depended on growth stage and *Nitrobacter* species studied.

With a change in nitrite concentration at constant pH, the undissociated nitrous acid concentration also varies. The influence of nitrite concentration, pH and nitrous acid concentration on the counting result of a soil sample was investigated. It was found that none of the three factors determined

the outcome exclusively. A certain optimum nitrous acid concentration was found, which possibly changes in time. Highest counting results were obtained in an incubation medium with a low nitrite concentration and a pH of 6.5.

Substrate inhibition was thought to be the cause of the low counting efficiency in Merrevliet samples. In Chapter 4 it is shown that this is true for the majority of the nitrite oxidizers present in this soil. The number of nitrite oxidizers in this water-saturated and thus probably oxygen-limited soil, was surprisingly high. The number of nitrite oxidizers greatly exceeded the number of ammonium oxidizers, which was confirmed by activity measurements. Because the number of nitrite oxidizers determined at the high nitrite concentration corresponded with the number of ammonia oxidizers, it was assumed that the MPN-technique applied discriminates between two subpopulation within the nitrite-oxidizing community. It is not known by what metabolism the nitrite sensitive cells that are capable of chemolithotrophic nitrite oxidation in MPN-series are growing in the soil itself. Several alternative possibilities are discussed. One of these presumed aerobic sites in this peat soil occurring around aerenchymatous plant roots. In this case, nitrite is most likely recycled by nitrate reduction. Chemolithotrophic nitrite oxidizers could also have been involved in this nitrate reduction as anaerobic growth of *Nitrobacter* is known to occur in the laboratory.

The observation that substrate inhibition plays a role in nitrite oxidizer growth and/or activity on the one hand, and the several reports in literature of very high K_m and K_s values combined with very low growth rates on the other hand, led to some theoretical considerations about the required length of the incubation period in media that differed in nitrite concentration. A computer model operating with Michaelis-Menten- and Monod-kinetics was designed to calculate the period needed to deplete the total amount of nitrite present in a MPN-incubation tube. Data on kinetic parameters were derived from the literature. The results of these modelled growth experiments are described in Chapter 5. The observed higher MPN-yields at high nitrite concentrations could be attributed to the fact that a 3 months incubation, which was in practice the maximum period applied, is not long enough for complete consumption of a low nitrite concentration for a number of combinations of kinetic parameters, whereas a higher concentration does not lead to underestimations of population sizes. Introducing a term for substrate inhibition of nitrifier growth, an underestimation of the numbers obtained at high nitrite concentration could also be demonstrated. An attempt was made to simulate a counting result of a Merrevliet soil sample incubated at several nitrite concentrations. A well fitted simulation could

only be obtained if there were two subpopulations present with quite different kinetics of nitrite oxidation. The model proved to be useful for the interpretation of MPN-counting results performed at different nitrite concentrations.

In Chapter 6, the temporal and spatial variation of different aspects of the nitrite-oxidizing community present in the Ommen-top soil is described, using different methods. The size of this community was determined applying the MPN-technique, its potential activity was established and specific fluorescent antibody enumerations of three serotypes of *Nitrobacter* gave insight into the changes in community composition. The amount of mineralizable organic nitrogen was determined as well as several abiotic soil parameters. Correlations between the numerous data were calculated. The saturation constants (K_m) for nitrite oxidation varied greatly as did the potential activity (V_{max}). However, the ratio V_{max}/K_m was relatively constant, indicating that the nitrite-oxidizing capacity at times with a low potential activity was compensated by a low K_m . Most probable numbers did not correlate with potential activities. This implies that the amount of viable cells and the total maximal activities are two different aspects of the nitrite-oxidizing community. Fluorescent antibody enumerations of three serotypes of *Nitrobacter* revealed a changing community composition and the coexistence of these serotypes in every soil sample.

Chapter 7 deals with the extent to which the kinetic parameters of *Nitrobacter winogradskyi* and *Nitrobacter hamburgensis* grown at different dilution rates in autotrophic and mixotrophic media could be influenced by environmental parameters such as the presence of organic compounds e.g. acetate, and dissolved oxygen concentration. Influenced by these two parameters, the K_m -value differed to the same extent as in the field study. The maximal nitrite-oxidizing activity per cell also varied with growth conditions, which explains the observed lack of correlation between the size of the population, as determined by the MPN-technique and its potential activity.

The observation that the three serotypes of *Nitrobacter* coexisted in soil samples of 400 grams, implies that a niche-differentiation between the serotypes takes place on a much smaller scale. However, it is not possible to make field observations of spatial distribution of nitrite oxidizers on a smaller scale. Therefore, a model system was developed that made direct observations possible of the spatial distribution on a μm -scale of bacteria around living plant roots using immunological techniques. This system and some results concerning the spatial distribution of *Nitrosomonas europaea*, *Nitrobacter winogradskyi* and a *Pseudomonas spec.* around the roots of *Plantago lanceolata* are described in Chapter 8. This model system proved

to be very convenient for the study of plant-bacteria relations. It showed a very strong interaction between the root and the *Pseudomonas spec.* No interaction could be found between young roots (0-6 days) and *N. europaea* or *N. winogradskyi*. Older roots had a significantly positive interaction with *N. europaea* but not with *N. winogradskyi*.